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Sp1-Dependent Regulation of the Tissue Inhibitor of Metalloproteinases-1 Promoter

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Abstract Extracellular matrix (ECM) remodeling is involved in many cellular properties such as division, migration, differentiation, and death. The turnover of ECM is regulated by matrix metalloproteinases (MMPs) and the MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMPs). In this study, the transcriptional regulation of the TIMP-1 promoter was investigated. The 5′-deletion assay showed that the region between −1,200 and −1,101 was responsible for the TIMP-1 promoter activity. The mutations of the two Sp1 sites in this region reduced the transcription activity. In addition, the co-transfection with antisense Sp1 oligonucleotide decreased the promoter activity, suggesting that the transcription of the TIMP-1 promoter is mediated by Sp1. Previously, it was reported that the TIMP-1 expression was enhanced under hypoxia. Therefore, the TIMP-1 promoter activity was investigated with or without cobalt ion, which elicits the same physiological effect as hypoxia. The results showed that the TIMP-1 promoter was induced in the presence of cobalt ion and that the promoter activity was regulated by Sp1 as well as HIF-1. Therefore, this study suggests that Sp1 is involved in the regulation of the TIMP-1 promoter in the presence of cobalt ion as well as in the basal level transcription. J. Cell. Biochem. 91: 1260–1268, 2004.

Key words: cobalt ion; Sp1; tissue inhibitor of metalloproteinases (TIMP)-1; transcription regulation

The interaction between extracellular matrix (ECM) and cells is important for many cellular properties such as division, migration, differentiation, and death [Lukashev and Werb, 1998]. Remodeling of the ECM occurs normally in embryonic development and wound healing [Raghow, 1994; Werb and Chin, 1998]. In addition, it is involved in the pathological processes such as rheumatoid arthritis [Cawston, 1998], restenosis [Batchelor et al., 1998], tumor invasion and metastasis [Lukashev and Werb, 1998; Sternlicht et al., 1999], arteriosclerosis [Newby and Zaltsman, 1999], and liver fibrosis [Friedman, 1993]. The matrix metalloproteinases (MMPs) are involved in the turnover of ECM [Reynolds, 1996; Werb and Chin, 1998]. The MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMPs) [Campbell et al., 1991; Edwards et al., 1996; Reynolds, 1996; Gomez et al., 1997]. Therefore, the balance between MMPs and TIMPs is important for normal turnover of ECM.

TIMP-1 is one of the TIMP family proteins. TIMP-1 has growth factor-like [Hayakawa et al., 1990, 1992] and anti-apoptotic properties [Boudreau et al., 1995; Alexander et al., 1996; Guedez et al., 1998a,b]. In addition, the expression of TIMP-1 is induced by growth factors, phorbol esters [Campbell et al., 1991], serum [Campbell et al., 1991], and viruses [Gewert et al., 1987; Coulombe et al., 1988]. It was previously shown that the induction of the TIMP-1 is mainly mediated by the enhanced transcription [Trim et al., 2000]. The previous reports showed that the TIMP-1 promoter is a TATA-less promoter [Coulombe et al., 1988].

Abbreviations used: AP-1, activating protein-1; LBP1, leader-binding protein 1; HIF-1, hypoxia inducible factor-1; PCR, polymerase chain reaction; PEA3, polyoma enhancer A3; Sp1, specificity protein 1; TRE, TPA-response element.

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It has been known that the transcription of the *TIMP-1* gene is regulated by various transcription factors including AP-1 [Uchijima et al., 1994; Logan et al., 1996], LBP1 [Clark et al., 1997], PEA3 [Edwards et al., 1992], and TRE like sequence [Edwards et al., 1992]. In addition, the sequence in exon1 and intron 1 was reported to have an important role in the transcription of the *TIMP-1* gene in fibroblasts [Dean et al., 2000]. The previous report showed that the activity of the TIMP-1 promoter was enhanced under hypoxia [Norman et al., 1999]. The hypoxia induction of the TIMP-1 promoter reached up to 1.5-fold after 48 h of incubation, compared to normoxia. A point mutation of hypoxia inducible factor-1 (HIF-1) binding site decreased the transcription of the *TIMP-1* gene under hypoxia, suggesting that the induction is mediated by HIF-1.

In the present study, we investigated the transcription regulation of the TIMP-1 promoter at a basal level and in the presence of cobalt ion. It was previously reported that cobalt ion has the same physiological effect as hypoxia [Maxwell et al., 1993; Wang and Semenza, 1993; Jiang et al., 1997; An et al., 1998; Chandel et al., 1998; Minchenko and Caro, 2000; Wang et al., 2000; Piret et al., 2002; Yuan et al., 2003]. The results showed that the transcription of the TIMP-1 promoter was enhanced in the presence of cobalt ion. Various analyses of the promoter suggest that the transcription of the TIMP-1 promoter is mediated by Sp1 in the presence of cobalt ion as well as at a basal level.

MATERIALS AND METHODS

Plasmids

The TIMP-1 promoter ($-1{,}730 \sim -931$) was cloned by PCR using the genomic DNA from HepG2 cells as a template. Nucleotides are numbered from the A residue of the translational initiation codon (ATG) in the second exon according to Campbell et al. [1991]. The genomic DNA was extracted from HepG2 cells by using Qiagen DNeasy Tissue system (Qiagen, Valencia, CA). The sequences of the specific primers for the TIMP-1 promoter were as follows; forward primer 5′-GAAGATCTAGAACCGGTACCCATCTCAG-3′, backward primer 5′-CCCAAGCTTCTGTACCTCTGGTGTCTCTC-3′. Bgl II and HinDIII sites were introduced to forward and backward primers respectively for the cloning convenience (the enzyme sites were underlined). The PCR amplified TIMP-1 fragment was digested with Bgl II and HinDIII and purified by gel electrophoresis and elution. The pGL3-promoter plasmid was purchased from Promega (Madison, WI). The SV40 promoter was eliminated by digestion with Bgl II and HinDIII and the plasmid backbone was purified by gel electrophoresis and elution. The TIMP-1 promoter fragment was inserted to pGL3-promoter, resulting in construction of pTIMP1-1730. The cloned TIMP-1 promoter was confirmed by DNA sequencing.

The deletion of the 5′-region of the promoter was made by PCR. The backward primer was same as above. The sequences of the forward primers were as follows; for TIMP1-1450, 5′-GAAGATCTTTTGTTTCCTCTCTGCCACC-3′: for TIMP1-1250, 5′-GAAGATCTGCCTCTAAGCTCTCGCTGAG-3′: for TIMP1-1200, 5′-GAAGATCTAGGTGGGGGAGGTGGCTGGC-3′: for TIMP1-1100, 5′-GAAGATCTGCTTCTGCACTGATGGTGGG-3′: for TIMP1-1006 5′-GAAGATCTGACATTTATCCTCTAGCGCT-3′. The PCR fragments were subcloned into pGL3-promoter as described above. The cloning of each fragment was confirmed by DNA sequencing.

The Sp1 mutant constructs were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using pTIMP1-1730 construct as a template. The sequences of the mutated Sp1 oligonucleotides used are as follows: Sp1 mutant A upstrand, 5′-CTCCTTTCGTCGGCCATAACCTTGGCTTCTGCAC-3′, Sp1 mutant A downstrand, 5′-GTGCAGAAGCCAAGGTTATGGCCGACGAAAGGAG-3′, Sp1 mutant B upstrand, 5′-GGCCCAGGGAGAGGGAGAGGATTATGGTGGGAGAGGA GGAGGG-3′, Sp1 mutant B downstrand, 5′-CCCTCCTCCTCTCCCACC ATAATCCTCTCCCTCTCCCTGGGCC-3′.

Cell Cultures and Transfection

The 293 human embryonic kidney cells were purchased from ATCC (Manassas, VA). The cells were maintained in DMEM supplemented with 10% FBS in a 5% CO_2 incubator. For the transfection assays, the cells were seeded at a density of 5.0×10^5 cells/well in 6-well flat-bottomed microassay plates (Falcon Co., Becton Dickenson, Franklin Lakes, NJ) 24 h before the transfection. Polyethylenimine (PEI, 25,000 Da) was used as a gene carrier. The plasmid/PEI complexes (2 μg plasmid/well) were prepared at

a 5/1 N/P ratio and incubated for 30 min at room temperature, based on the previous reports [Abdallah et al., 1996; Lemkine et al., 1999; Turunen et al., 1999; Wightman et al., 1999; Nguyen et al., 2000; Lee et al., 2003]. The cells were washed twice with serum-free medium, and then 2 ml of fresh serum-free medium was added. The plasmid/PEI complex was added to each well. The cells were then incubated for 4 h at 37°C in a 5% CO_2 incubator. After 4 h, the transfection mixtures were removed and 2 ml of fresh medium containing FBS was added. The cells were incubated at the desired concentration of oxygen or $CoCl_2$ for 20 h. The cells were harvested for luciferase assay.

For co-transfection with the antisense oligonucleotides, the DNA/PEI complexes (2 μg oligonucleotides/well and 2 μg plasmid/well) were prepared at a 5/1 N/P (nitrogen atom in PEI/phosphate in plasmid) ratio. The transfection assay was carried out as described above. The antisense oligonucleotides were synthesized according to the previous report [Comerford et al., 2002]. The sequences of the oligonucleotides are as follows: antisense Sp1, 5′-ATATTAGGCATCACTCCAGG-3′; sense Sp1, 5′-CCTGGAGTGATGCCTAATAT-3′; antisense HIF-1, 5′-GCCGGCGCCCTCCAT-3′; sense HIF-1, 5′-ATGGAGGGCGCCGGC-3′.

Luciferase Assay

After incubation, the cells were washed with PBS twice, and 0.15 ml of reporter lysis buffer (Promega) was added to each well. After 15 min of incubation at room temperature, the cells were harvested and transferred to microcentrifuge tubes. After 15 s of vortexing, the cells were centrifuged at 11,000 rpm for 3 min. The extracts were transferred to fresh tubes and stored at −70°C until use. The protein concentrations of the extracts were determined by using a BCA protein assay kit (Pierce, Iselin, NJ). Luciferase activity was measured in terms of relative light units (RLU) using a 96-well plate Luminometer (Dynex Technologies, Inc., Chantilly, VA). The luciferase activity was monitored and integrated over a period of 60 s. The final values of luciferase were reported in terms of RLU/mg total protein.

Statistical Analysis

The comparison of the luciferase activity was made by Student's *t*-test. *P* value under 0.05 was thought to be statistically significant.

RESULTS

Deletion Analysis of the RTP801 Promoter

To identify the region necessary for the transcriptional regulation in the TIMP-1 promoter, various fragments of the 5′-flanking region of the TIMP-1 promoter were cloned into the luciferase reporter gene plasmid (Fig. 1A). These constructs were transfected into the 293 cells using PEI as a gene carrier. After 20 h of the incubation at 37°C, the cell extracts were prepared from the transfected cells and the luciferase activity was measured. The luciferase activity of pTIMP1-1100 was decreased significantly, compared to that of pTIMP1-1200 (Fig. 1B). This result indicates that the cis-regulatory elements for the regulation of the TIMP-1 promoter exist between −1,200 and −1,101.

Role of Sp1 in the Transcription of the TIMP-1 Promoter

Sequence analysis showed that there were two potential Sp1 consensus binding sites in the region between −1,200 and −1,101 (Fig. 2A). To evaluate the effect of mutation of these Sp1 binding sites, site-directed mutagenesis was performed. The sequence of GGCG (−1,151 ∼ −1,148) was replaced with the sequence of TTAT, resulting in construction of pTIMP1-SP1(−)A (Fig. 2A). Similarly, the sequence of CGCC (−1,109 ∼ −1,106) was replaced with the sequence of ATAA, resulting in construction of pTIMP1-SP1(−)B. In pTIMP1-SP1(−)AB, both the Sp1 binding sites were mutated. pTIMP1-1730 (wild-type), pTIMP1-Sp1(−)A, pTIMP1-Sp1(−)B, and pTIMP1-Sp1(−)AB were transfected into the 293 cells. After 20 h of the incubation at 37°C, the luciferase activity was measured. In the cells transfected with pTIMP1-Sp1(−)A and pTIMP1-Sp1(−)B, the luciferase activity was decreased, compared to the cells transfected with wild-type plasmid (Fig. 2B). Furthermore, the cells transfected with pTIMP1-Sp1(−)AB showed the lowest luciferase activity (Fig. 2B). These results showed that both the Sp1 sites were important for the activity of the TIMP-1 promoter.

Another approach to show that Sp1 is important for the transcriptional regulation of the TIMP-1 promoter was to use antisense oligonucleotides directed against Sp1 (Fig. 3). The antisense Sp1 oligonucleotides

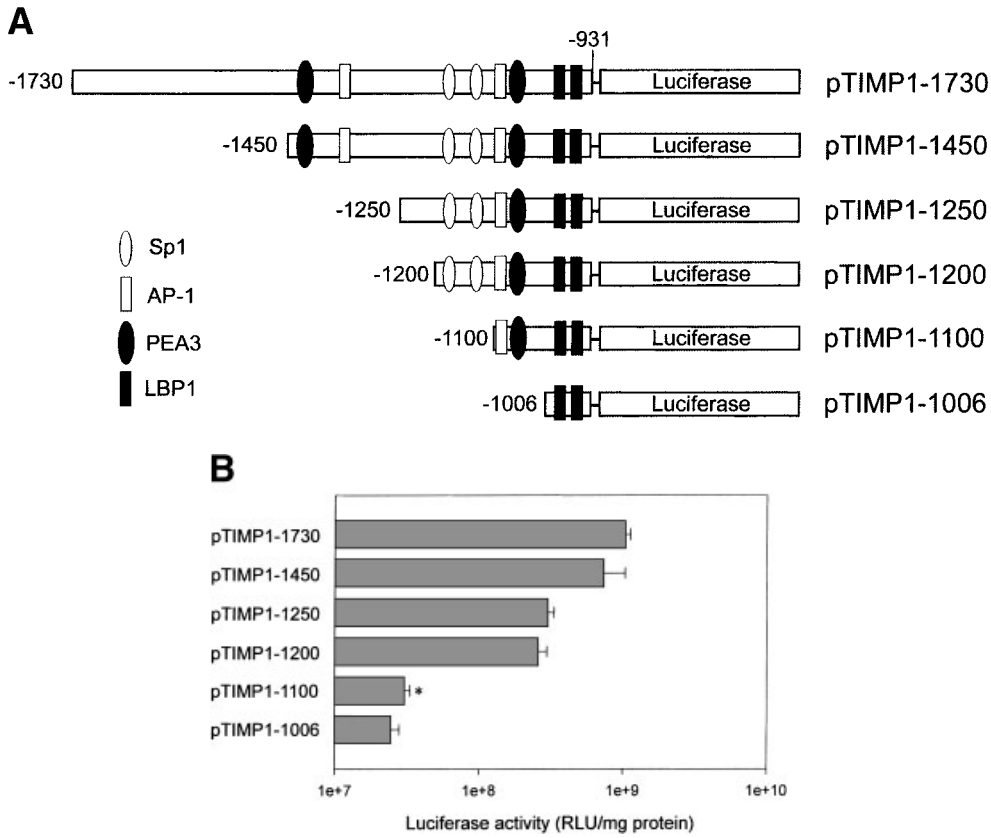

Fig. 1. **A**: The structures of the pTIMP-1 luciferase reporter vectors. The diagram shows the structures of the pTIMP-1 luciferase reporter vectors containing various lengths of 5′-flanking regions of the TIMP-1 promoter. Consensus sequences for transcription factor binding are presented. **B**: Promoter activity of the 5′-flanking region of the TIMP-1 promoter. The reporter constructs were transiently transfected into the 293 cells. The cell extracts were prepared from the cells and the luciferase activity was measured. The data is expressed as mean values (±standard deviation) of four experiments. $*P < 0.01$ as compared to pTIMP1-1730, pTIMP1-1450, pTIMP1-1250, pTIMP1-1200, but no statistical significance as compared to pTIMP1-1006.

were co-transfected with pTIMP1-1730 into the 293 cells to reduce the expression of Sp1. As a control, the sense Sp1 oligonucleotides were co-transfected with pTIMP1-1730. As a result, in the cells transfected with the antisense Sp1 oligonucleotides, the activity of the TIMP-1 promoter was decreased, compared to the sense Sp1 oligonucleotides transfected cells (Fig. 3). This result suggests that Sp1 mediates the transcription of the TIMP-1 promoter.

Role of Sp1 in the Transcriptional Induction of the TIMP-1 Promoter in the Presence of Cobalt Ion

It was previously reported that the TIMP-1 expression was induced under hypoxia [Norman et al., 1999]. To confirm the hypoxia induction of the TIMP-1 promoter, we incubated the cells under hypoxia or normoxia after transfection. The results showed that the promoter activity was induced under hypoxia (Fig. 4A). Cobalt ion has the same physiological effect as hypoxia to cells [Maxwell et al., 1993; Wang and Semenza, 1993; Jiang et al., 1997; An et al., 1998; Chandel et al., 1998; Minchenko and Caro, 2000; Wang et al., 2000; Piret et al., 2002; Yuan et al., 2003]. Therefore, we incubated the transfected cells with cobalt ion to evaluate the transcriptional induction of the TIMP-1 promoter. After 20 h of the incubation with 100 μM $CoCl_2$, the luciferase expression was induced by about three times (Fig. 4B). To evaluate the mutations of the Sp1 sites, pTIMP1-1730 (wild-type), pTIMP1-Sp1(−)A, pTIMP1-Sp1(−)B, and pTIMP1-Sp1(−)AB were transfected into the 293 cells and the transfected cells were incubated with 100 μM $CoCl_2$ for 20 h. The results showed that the mutations of the Sp1 sites decreased the transcriptional activity of the TIMP-1 promoter (Fig. 4B). In

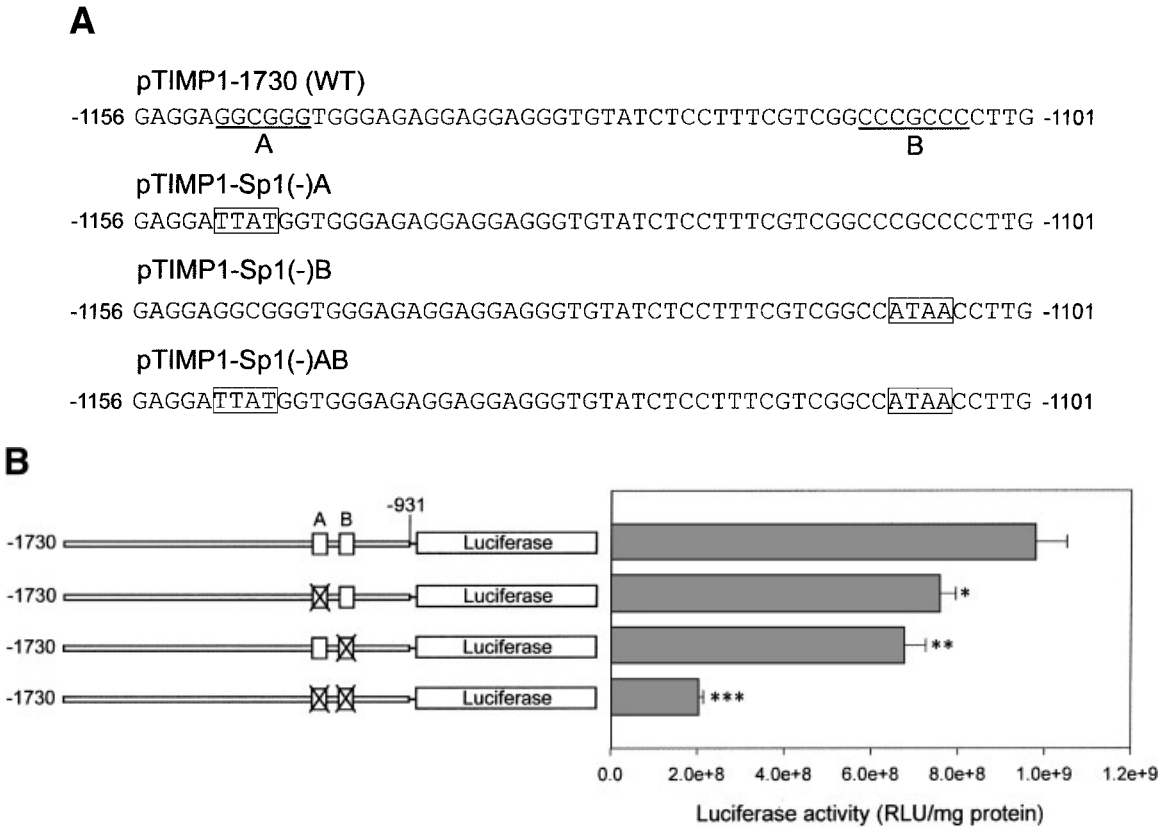

Fig. 2. **A**: Sequence of the TIMP-1 promoter between −1,156 and −1,101. The Sp1 consensus sequences of the TIMP-1 promoter in the pTIMP1-1730 are underlined. The sequences of the pTIMP1-Sp1(−)A, pTIMP1-Sp1(−)B, and pTIMP1-Sp1(−)AB are also shown. The mutation positions are indicated by rectangles. **B**: Effects of mutations in the Sp1 elements in the TIMP-1 promoter on the promoter activity. pTIMP1-1730, pTIMP1-Sp1(−)A, pTIMP1-Sp1(−)B, and pTIMP1-Sp1(−)AB were transiently transfected into the 293 cells. The cell extracts were prepared from the cells and the luciferase activity was measured. The data is expressed as mean values (±standard deviation) of four experiments. *$P < 0.01$ as compared to pTIMP1-1730 and pTIMP1-Sp1(−)AB, but no statistical significance as compared to pTIMP1-Sp1(−)B. **$P < 0.01$ as compared to pTIMP1-1730 and pTIMP1-Sp1(−)AB. ***$P < 0.01$ as compared to pTIMP1-1730.

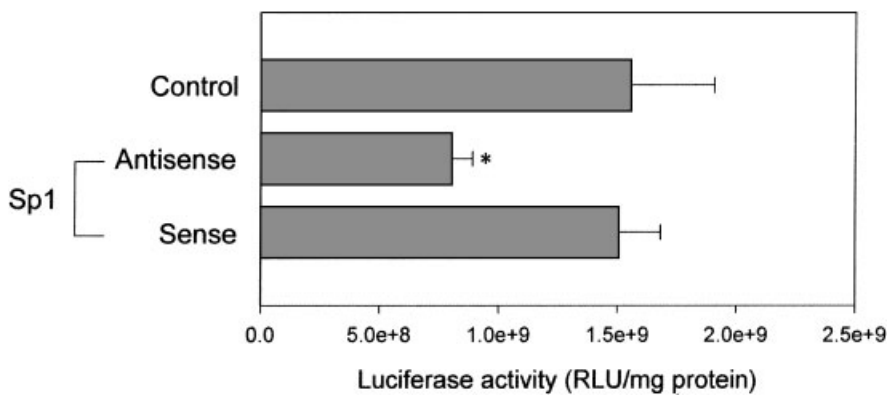

Fig. 3. Role of Sp1 in the TIMP-1 promoter. pTIMP1-1730 was co-transfected into the 293 cells with Sp1 sense or Sp1 antisense oligonucleotides. The cell extracts were prepared from the cells and the luciferase activity was measured. The data is expressed as mean values (±standard deviation) of four experiments. *$P < 0.01$ as compared to control and Sp1 sense oligonucleotide.

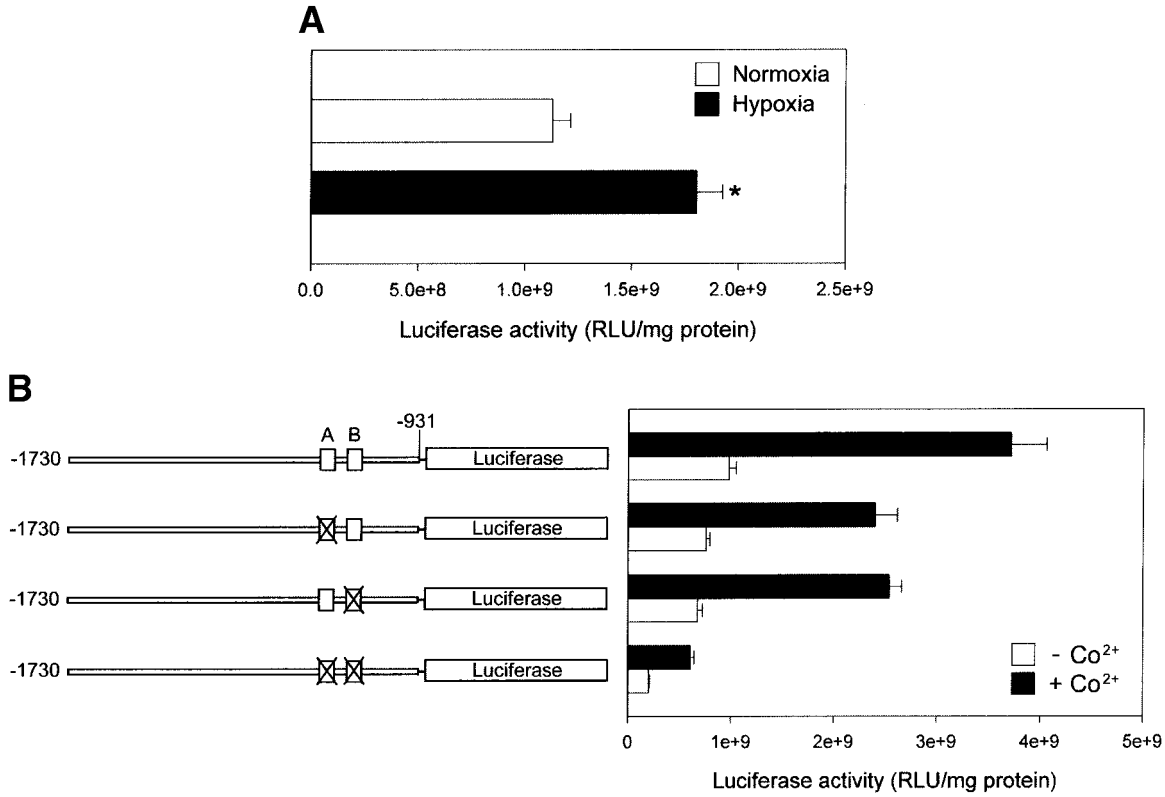

Fig. 4. **A**: Induction of the TIMP-1 promoter under hypoxia. pTIMP1-1730 was transiently transfected into the 293 cells. The cells were incubated for 20 h under hypoxia (1% oxygen) or normoxia (20% oxygen). After the incubation, the luciferase activity was measured. The data is expressed as mean values (±standard deviation) of four experiments. $*P < 0.01$ compared to normoxia. **B**: Effects of mutations in the Sp1 elements in the induction of the TIMP-1 promoter in the presence of cobalt ion. pTIMP1-1730, pTIMP1-Sp1(−)A, pTIMP1-Sp1(−)B, and pTIMP1-Sp1(−)AB were transiently transfected into the 293 cells. The cells were incubated for 20 h with or without cobalt ion. After the incubation, the luciferase activity was measured. The data is expressed as mean values (±standard deviation) of four experiments.

addition, the two Sp1 sites were equally important in the transcription of the TIMP-1 promoter in the presence of cobalt ion. These results suggest that the two Sp1 sites are involved in the transcriptional induction of the TIMP-1 promoter in the presence of cobalt ion.

To confirm that the transcription is induced by Sp1, we co-transfected the antisense Sp1 oligonucleotides with pTIMP1-1730. The transfected cells were incubated with or without cobalt ion for 20 h. As a result, the activity of the TIMP-1 promoter was decreased significantly in the cells co-transfected with the antisense Sp1 oligonucleotides (Fig. 5). In the previous report, it was suggested that HIF-1 might be important in the induction of the TIMP-1 promoter under hypoxia. Therefore, we co-transfected pTIMP1-1730 with the antisense HIF-1 oligonucleotides into the 293 cells. The cells were incubated with cobalt ion for 20 h. The results showed that antisense HIF-1 oligonucleotides decreased the transcription of the TIMP-1 promoter (Fig. 5). Therefore, Sp1 and HIF-1 may be involved in the transcriptional induction of the TIMP-1 promoter.

DISCUSSION

In the present study, we have shown that the TIMP-1 promoter is regulated by the two Sp1 sites at −1,146 and −1,105. The 5′-deletion assay showed that the region between −1,200 and −1,101 was responsible for the TIMP-1 promoter activity. This region contained two Sp1 consensus sequences. The mutation analysis, in which each Sp1 site was disrupted, showed that the transcriptional activity of the TIMP-1 promoter was significantly decreased. When both the Sp1 sites were mutated, the promoter activity was further decreased, suggesting both the Sp1 sites are important for the transcriptional regulation of the TIMP-1

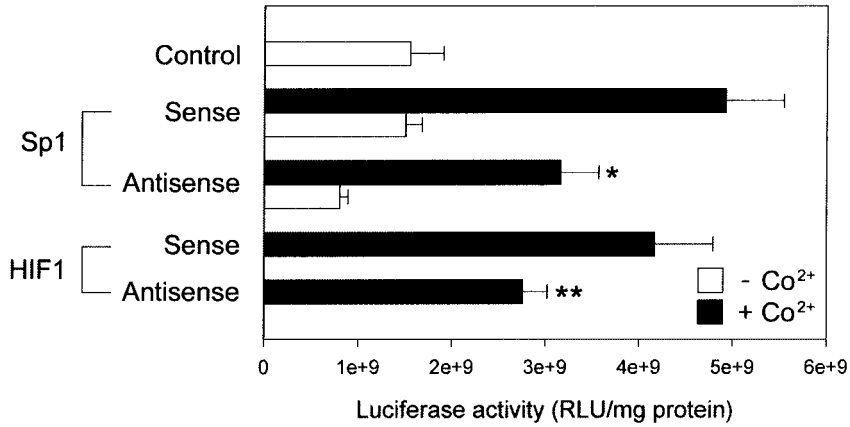

Fig. 5. Role of Sp1 in the induction of the TIMP-1 promoter in the presence of cobalt ion. pTIMP1-1730 was co-transfected into the 293 cells with Sp1 sense, Sp1 antisense, HIF-1 sense, or HIF-1 antisense oligonucleotides. The cells were incubated with or without cobalt ion for 20 h and assessed for luciferase activity. The data is expressed as mean values (±standard deviation) of four experiments. $*P < 0.01$ as compared to the Sp1 sense oligonucleotide. $**P < 0.01$ as compared to the HIF-1 sense oligonucleotide.

promoter. Furthermore, in the cells transfected with the antisense Sp1 oligonucleotides, the activity of the TIMP-1 promoter was reduced. These results suggest that Sp1 plays an important role at a basal level transcription of the TIMP-1 promoter.

The transcription of TIMP-1 was enhanced by tissue hypoxia in the KF cells [Norman et al., 1999]. It was suggested that the transcriptional induction of the TIMP-1 promoter in the KF cells was mediated by HIF-1. In present study, we incubated the transfected cells with cobalt ion to evaluate the transcriptional induction of the TIMP-1 promoter. It was previously reported that cobalt ion has the same physiological effect to the cells as hypoxia [Maxwell et al., 1993; Wang and Semenza, 1993; Jiang et al., 1997; An et al., 1998; Chandel et al., 1998; Minchenko and Caro, 2000; Wang et al., 2000; Piret et al., 2002; Yuan et al., 2003]. After 24 h of the incubation, the transcriptional activity of the promoter increased compared to the cells without cobalt ion, confirming the previous report (Fig. 4A,B) [Norman et al., 1999]. The antisense HIF-1 oligonucleotides co-transfection study also proved that HIF-1 was one of the key transcription factors for the TIMP-1 promoter. However, in the cells transfected with the antisense Sp1 oligonucleotides, the activity of the TIMP-1 promoter was significantly decreased. Therefore, these results indicated that Sp1 was another important transcription factor of the TIMP-1 promoter in the presence of cobalt ion. The role of Sp1 under hypoxia was previously suggested by Sanchez-Elsner et al. [2002]. In the endoglin promoter, Sp1 formed multi-protein complex with Smad and HIF-1 [Sanchez-Elsner et al., 2002]. This indicates that Sp1 and HIF-1 may cooperate to induce the gene expression under hypoxia. In addition, Xu et al. [2000] suggested that the cyclooxygenase-2 (cox-2) promoter was induced by Sp1 in hypoxic vascular endothelium. The Sp1 binding to the cox-2 promoter increased in response to hypoxia [Xu et al., 2000]. Another example of the Sp1 mediated hypoxia induction is the RTP801 promoter [Lee et al., 2004]. The RTP801 promoter was induced under hypoxia about five times compared to normoxia. It was suggested that this induction might be regulated by HIF-1 [Shoshani et al., 2002]. However, the induction of the RTP801 promoter was also regulated by Sp1 binding to the promoter [Lee et al., 2004]. Therefore, these reports suggested that Sp1 was an important transcriptional factor in the inductions of the various promoters under hypoxia.

In summary, the deletion and mutation analyses showed that the two Sp1 motifs are required for the transcription of the *TIMP-1* gene. In addition, the co-transfection assay with the antisense oligonucleotides suggests that Sp1 plays an important role in the transcription of the *TIMP-1* gene. The activity of the TIMP-1 promoter was induced in the presence of cobalt ion. The mutation analysis and the co-transfection assay with the antisense oligonucleotides suggest that Sp1 is involved in the transcription

of the *TIMP-1* gene in the presence of cobalt ion. Therefore, the transcription of the *TIMP-1* gene may be mediated by Sp1 in the presence of cobalt ion as well as at a basal level.

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